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Mitogenesis in Response to PDGF and Bombesin Abolished by Microinjection of Antibody to PIP_2

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The turnover of phosphatidylinositol 4,5-bisphosphate (PIP_2) is believed to constitute a crucial step in the signaling pathways for stimulation of cells by a variety of bioactive substances, including mitogens, but decisive evidence for the idea has not been obtained. In the present study, a monoclonal antibody to PIP_2 was microinjected into the cytoplasm of NIH 3T3 cells before or after exposure to mitogens. The antibody completely abolished nuclear labeling with [^{3}H]thymidine induced by platelet-derived growth factor and bombesin, but not by fibroblast growth factor, epidermal growth factor, insulin, or serum. The findings strongly suggest that PIP_2 breakdown is crucial in the elicitation and sustaining of cell proliferation induced by some types of mitogens such as platelet-derived growth factor and bombesin.

MUCH ATTENTION HAS BEEN PAID to inositol phospholipids recently because of their crucial, although so far indecisive, roles in transmembrane signal transduction (*1*). Treatment of cells with mitogens such as platelet-derived growth factor (PDGF) and bombesin rapidly elicits an enzymatic hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (*2*), both intracellular second messengers (*1, 3*). Moreover, inositol phospholipid turnover has been reported to be enhanced in cells transformed by tumor viruses and chemical carcinogens (*4*). PIP_2 breakdown is therefore believed to trigger cell proliferation. However, direct evidence to prove this has not been provided. In addition, mitogens such as fibroblast growth factor (FGF) and insulin do not elicit PIP_2 breakdown (*5–7*). Epidermal growth factor (EGF) is also considered not to evoke PIP_2 breakdown (*6, 7*) except in some cells such as A431 epidermoid carcinoma cells (*8*). These findings raise the question of whether PIP_2 breakdown is an indispensable process for the promotion of cell proliferation. Our aim in the present study, in which an antibody to PIP_2 was microinjected, was to establish the presumed involvement of PIP_2 breakdown in the signaling pathways of cell proliferation. We showed that inhibition of PIP_2 breakdown by the antibody abolishes the stimulation of quiescent cells caused by some types of mitogen.

For development of the antibody to PIP_2, BALB/c mice were immunized with PIP_2 from bovine spinal cords, and hybridoma cells were prepared by a conventional method (*9*). Among the cells, a clone secreted an antibody of the immunoglobulin G2b class. The immunoglobulin G (IgG) produced by this clone (designated antibody kt3g) was purified and examined for its affinity for lipids (Fig. 1a). Treatment with antibody kt3g resulted in a pattern identical with that of a control (treated with IgG from an unimmunized mouse), except for stained PIP_2. Therefore antibody kt3g was found to bind to PIP_2 and to have virtually no specific affinity for other lipids. Such specific binding of antibody kt3g to PIP_2 was further confirmed by an enzyme-linked immunosorbent assay (*10*). The results also showed that antibody kt3g did not cross-react with IP_3 and that it had a weak but detectable affinity for phosphatidylinositol 4-phosphate (PIP). In order to examine effects of antibody kt3g on the turnover of PIP_2 and PIP, we treated a cell membrane preparation with the antibody and determined the changes in the amounts of endogenous PIP_2 and PIP during a subsequent incubation. Antibody kt3g strongly suppressed the decrease in the membranous PIP_2 (Fig. 1b). This effect appeared to depend on the dose of antibody kt3g. Treatment with IgG from an unimmunized mouse had no effect, and antibody kt3g exerted little effect on the turnover of PIP. These results indicate that antibody kt3g specifically interacts with PIP_2 in the membrane and suppresses its turnover. The reason for the antibody effect on PIP_2 turnover is not clear, but it is conceivable that binding of antibody kt3g to PIP_2 would mask the reaction site for phospholipase C, the key enzyme for PIP_2 turnover (*1*), and this would lead to interference with the action of phospholipase C on PIP_2. Indeed, the hydrolysis of PIP_2 in an in vitro assay system using purified phospholipase C (*11*) was decreased by 92.4 ± 2.4% after preincubation of PIP_2 with one-fourth the equivalent of antibody kt3g, whereas antibody kt3g reduced the hydrolysis of PIP in a parallel assay only by 18.1 ± 12.0% (*12*). Therefore, it is likely that antibody kt3g introduced into cells by microinjection would also specifically block PIP_2 breakdown.

NIH 3T3 cells (clone 5611 from the Japanese Cancer Research Resources Bank) were inoculated onto glass cover slips at a density of 5×10^3 to 1×10^4 cell/cm^2 in the presence of 10% calf serum. Quiescence was achieved by culturing the cells in a serum-free medium supplemented with transferrin (human, 5 μg/ml) and albumin (bovine serum fraction V, 0.5 mg/ml) for 24 to 36 hours (*13*). Microinjection was then performed as follows. Antibody kt3g and the control IgG were prepared as solutions (0.1 to 5 mg/ml) in phosphate buffer (140 m*M* K^+, *p*H 7.25) and injected into the cytoplasm of cells with an injectoscope (Olympus, model IMT-2-SYF) (*14*). An attempt was made to inject all of the cells in certain compartments on cover slips delimited by scratches of a diamond pen. The cells were washed and incubated for 2 hours in the medium described above. After addition of mitogens, the cells were cultured in the presence of [^{3}H]thymidine (1 μCi/ml) for the indicated period and sampled for autoradiography (*15*) to determine the labeling indices (the percentages of the cells that incorporated [^{3}H]thymidine) of both the injected and uninjected cells.

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When NIH 3T3 cells injected with antibody kt3g (2 mg/ml) were exposed to PDGF (0.5 unit/ml) and sampled 17 hours later, only a small proportion of cells in the injected area were labeled with [^{3}H]thymidine as compared with cells in the uninjected area of the cover slips. The effect of antibody kt3g on S-phase entry of PDGF-exposed cells was further specified. Microinjection of antibody kt3g at various concentrations showed that the antibody inhibition of PDGF-induced nuclear labeling was dose dependent (Fig. 2a). Since antibody kt3g at concentrations higher than 2 mg/ml completely inhibited the mitogenic effect of PDGF, the concentration of antibody kt3g was fixed at 2 mg/ml in the subsequent experiments. The control IgG from an unimmunized mouse, when injected at 2 mg/ml into NIH 3T3 cells, caused little, if any, inhibition of PDGF-induced mitogenesis (Fig. 2a) and thus eliminated the possibility that the action of antibody kt3g on cell proliferation is attributable to artifacts accompanying the process of microinjection.

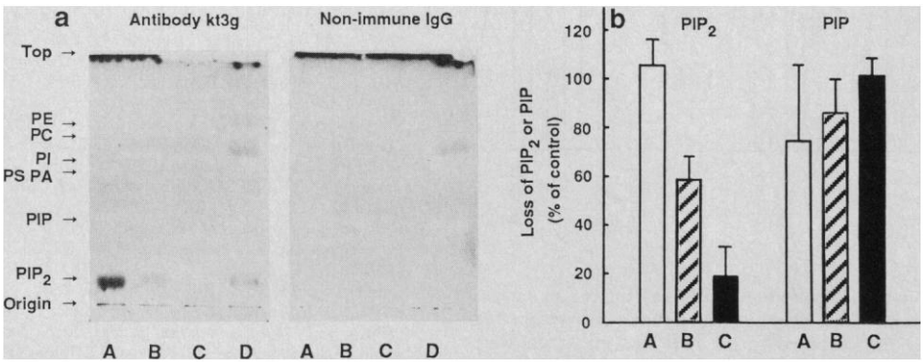

Fig. 1. Specificity of antibody kt3g. (**a**) Affinity for PIP_2. Thin-layer chromatography–immunostaining assay was performed as reported (*19*). A mixture of PIP_2, PIP, and phosphatidylinositol (PI) [(A) 1 μg, (B) 0.1 μg, and (C) 0.01 μg each per spot] and total lipid extract from rat brains [(D) 20 μg lipids per spot] were developed on thin-layer plates and treated with 10 μg/ml of antibody kt3g (left) or the control mouse IgG (right), followed by peroxidase staining. Authentic lipids on separate lanes were visualized in iodine vapor. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. (**b**) Effect on PIP_2 breakdown. Membranes of rat pituitary GH_3 cells labeled with myo-[^{3}H]inositol for 72 hours were prepared and degradation of inositol phospholipids was assayed essentially by the method of Jackowski *et al.* (*20*). Aliquots of the membrane preparation each containing 4.4 pmol (42,000 dpm) of inositol phospholipids (PIP_2, 3.3%; PIP, 15.8%) were suspended in 0.1*M* tris-HCl, *p*H 7.0, 2 m*M* Mg^{2+}, 200 μ*M* Ca^{2+}, and 100 μ*M* guanosine triphosphate (GTP) and incubated on ice for 2 hours with vehicle (control), IgG from an unimmunized mouse [(A) 200 μg/ml finally], or antibody kt3g [(B) 20 μg/ml and (C) 200 μg/ml finally]. The mixtures were then incubated at 37°C for 10 minutes, and decreases in the contents of PIP_2 and PIP were determined by a combination of thin-layer chromatography and liquid scintillation counting. Results were normalized by taking values for control samples (PIP_2, 27.3 fmol per assay; PIP, 156 fmol per assay) as 100% (mean and standard error of triplicate samples).

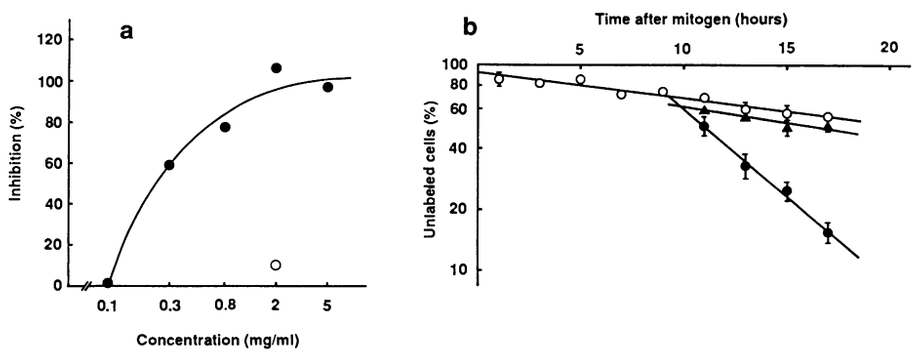

Fig. 2. Effect of antibody to PIP_2 on PDGF-induced S-phase entry. (**a**) Dose-dependence. NIH 3T3 cells were cultured on glass cover slips in a serum-free medium supplemented with transferrin (5 μg/ml) and albumin (0.5 mg/ml) for 24 to 36 hours. Antibody kt3g (●) or the control IgG (○) at the indicated concentrations was microinjected into the cytoplasm of the cells as described in the text and, 2 hours later, PDGF (0.5 unit/ml) or vehicle was added to the medium. The cells were cultured for an additional 17 hours in the presence of [^{3}H]thymidine (1 μCi/ml) and processed for autoradiography (*15*). The labeling indices (the percentages of the cells labeled with [^{3}H]thymidine) were determined by examining about 100 injected cells and more than 200 uninjected cells on each cover slip under a microscope. Inhibition was calculated as the microinjection-induced decrease in the labeling index of PDGF-exposed cells divided by the PDGF-induced increase in the labeling index of uninjected cells, each measurement based on two to six samples. The labeling indices of PDGF-exposed and unstimulated cells were virtually the same as those in Fig. 3a, and standard errors of the labeling indices ranged from 0.6 to 4.7%. (**b**) Time course. NIH 3T3 cells injected with antibody kt3g (2 mg/ml) and then exposed to PDGF and [^{3}H]thymidine were harvested at intervals and analyzed by autoradiography as described above. The data are plotted by the method of Smith and Martin (*21*). (○) Unstimulated cells; (●) PDGF-exposed cells; (▲) cells injected with antibody kt3g and then exposed to PDGF. Short bars indicate standard errors of two to five samples. The labeling index of PDGF-treated cells up to 10 hours was not examined, but other control experiments showed that there was no change in the labeling index up to 9 hours after exposure to PDGF.

The above finding was confirmed in a time course experiment, in which uninjected cells and cells injected with antibody kt3g were exposed to PDGF and sampled at intervals (Fig. 2b). Exposure of the cells to PDGF increased the transition rate (the rate of S-phase entry) 6.5-fold after about 9.5 hours. However, when cells were injected with antibody kt3g before PDGF treatment, the transition rate of PDGF-exposed cells was virtually reversed to the level of unstimulated cells, irrespective of the time after exposure to PDGF.

A question of interest is whether such an effect of antibody kt3g on cell proliferation is specific to PDGF-induced mitogenesis or not. To test this, cells microinjected with antibody kt3g were exposed to various mitogens (Fig. 3a). Injection with antibody kt3g completely reversed the labeling index of the cells exposed to PDGF or bombesin to the background level of unstimulated cells. Mitogenic effects of PDGF and bombesin in combination with insulin were also abolished by antibody kt3g. In contrast, when cells injected with antibody kt3g were exposed to serum, FGF, EGF, insulin, or a combination of FGF and insulin, there was little, if any, difference in the labeling index between the injected and uninjected cells. These results show that there are at least two distinct signaling pathways—one sensitive and the other insensitive to the injected antibody.

Concerning the action of antibody kt3g injected into cells, the antibody would bind to endogenous PIP_2 specifically, leading to inhibition of its hydrolysis by phospholipase C (Fig. 1). Although it may also be possible that binding of the antibody to PIP_2 affects another cellular event indirectly (for example, by changing membrane structure), the event must be crucial for PDGF- and bombesin-induced mitogenesis and must not be involved in mitogenesis by FGF, EGF, and insulin. The existence of such an event is, so far, unclear. Another possibility is that antibody kt3g also acts on PIP, since hydrolysis of PIP as well as PIP_2 results in the forma-

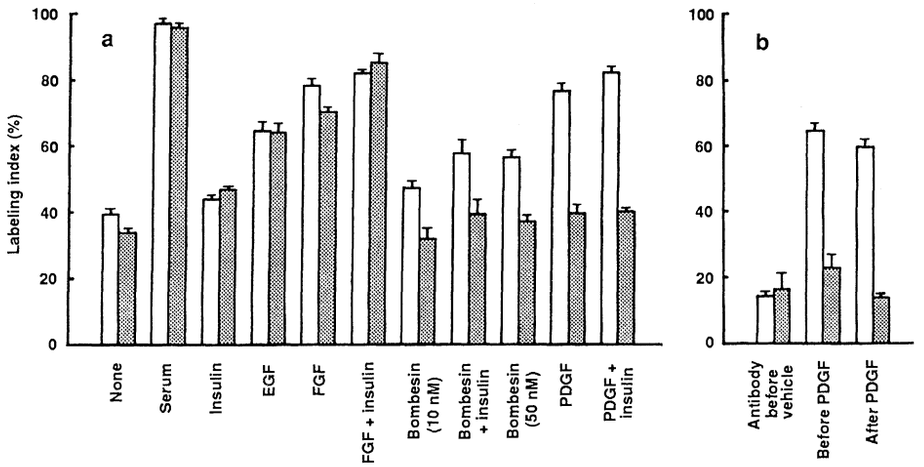

Fig. 3. Changes in S-phase entry due to microinjection of antibody to PIP_2. (**a**) Effects on various mitogenic stimuli. The labeling indices of NIH 3T3 cells, which were uninjected (open bars) or injected with 2 mg/ml of antibody kt3g (shaded bars) and then exposed to the indicated mitogens, were determined as described in the legend to Fig. 2 (mean ± standard error of three to four samples). Injection of the control IgG (2 mg/ml) caused little change in the labeling index for any treatment. None, vehicle; serum (calf), 5%; insulin, 10 μg/ml; EGF, 50 ng/ml; FGF (pituitary), 50 ng/ml; bombesin, 10 n*M* or 50 n*M*; PDGF, 0.5 unit/ml. (**b**) Difference in the antibody effect between stimulated and unstimulated cells. NIH 3T3 cells were cultured in a serum-free medium supplemented with transferrin, albumin, and insulin (1 μg/ml) for 18 to 24 hours and then exposed to PDGF (1 unit/ml) or vehicle. [^{3}H]thymidine was added 3 hours after addition to PDGF. The cells were cultured for an additional 14 hours and sampled for autoradiography. Antibody kt3g (2 mg/ml) was injected 2 hours before or after PDGF treatment. The labeling indices of the cells uninjected (open bars) and injected (shaded bars) were determined as described in the legend to Fig. 2 (mean ± standard error of three samples).

tion of 1,2-diacylglycerol (*1*), and involvement of PIP breakdown in thrombin-induced mitogenesis has been suggested (*16*). However, antibody kt3g has very little ability to bind to PIP and to suppress PIP turnover as compared with PIP_2 (Fig. 1) and, therefore, such an interpretation does not apply to our results. Consequently, it is most likely that the prevention of S-phase entry by the injection of antibody kt3g is caused by its inhibition of PIP_2 breakdown.

In support of the idea, only the mitogenesis by growth factors eliciting PIP_2 breakdown [PDGF and bombesin (*2*)] was sensitive to the antibody to PIP_2. Serum, which elicits PIP_2 breakdown (*6, 7, 17*), promoted mitogenesis even after the antibody injection. However, since serum contains a variety of mitogenic substances, it is conceivable that the mitogenic effect of serum is mainly caused by PIP_2 turnover-independent mitogens, as suggested by Besterman *et al.* (*6*). Therefore, the present findings strongly suggest that PIP_2 breakdown is a crucial step in the signaling pathways of cell proliferation only promoted by the mitogens eliciting PIP_2 breakdown.

Another question that can be raised is whether a single burst of PIP_2 turnover suffices or its continuance is requisite for the promotion of cell proliferation by mitogens such as PDGF and bombesin. It has been reported that, according to the competence-progression model (*18*), cells once exposed to PDGF remain "competent" for more than 10 hours after removal of PDGF. To test the alternatives, we performed an experiment in which serum-deprived cells were maintained in the presence of insulin, a progression factor, and then antibody to PIP_2 was injected into uncommitted cells before PDGF stimulation or into committed cells after PDGF stimulation (Fig. 3b). Injection of antibody kt3g completely reversed PDGF-induced S-phase entry of both the cells, committed or uncommitted, to the level of the control cells (supplied with insulin only). This result indicates that continual PIP_2 breakdown is requisite to sustain PDGF-induced mitogenesis. We now speculate as follows: Exposure of cells to PDGF elicits activation of phospholipase C, which hydrolyzes PIP_2, leading to subsequent elevation of cytosolic Ca^{2+} concentration and activation of protein kinase C, and results finally in cell proliferation (*1, 3*). The phospholipase C once activated would remain active during subsequent hours, even after removal of PDGF, hence the maintenance of competence. Injection of antibody to PIP_2 would, however, block the hydrolysis of PIP_2 and result in the prevention of mitogenesis.

In conclusion, a cellular event (or events) sensitive to antibody to PIP_2, most likely PIP_2 breakdown by phospholipase C, appears to be a crucial process in the initiation and maintenance of cell proliferation induced by some types of mitogens such as PDGF and bombesin; mitogenesis by FGF, EGF, insulin, and serum is independent of such an event.

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Cell-Cell Interactions in the Guidance of Late-Developing Neurons in *Caenorhabditis elegans*

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The initial outgrowth of developing neuronal processes can be affected by a number of extrinsic interactions. Cell-cell interactions are also important in a later stage of neuronal outgrowth when processes grow into the region of their targets. The correct positioning of the process of a postembryonic sensory neuron, the touch cell AVM of the nematode *Caenorhabditis elegans*, at its synaptic targets requires the presence of a pair of embryonic interneurons, the BDU cells. These cells receive synapses from AVM but do not participate in the touch reflex circuit. Therefore, the AVM-BDU synapses may be required to stabilize the association between these cells and assist in the guidance of the AVM processes through a mature neuropil.

CUES PROVIDED BY NEURONS (*1, 2*), glia, other associated cells (*1, 3*), and components of the extracellular matrix (*4*) influence the initial outgrowth of neuronal processes (*5*). Much less is known about the final stages of neuronal development, when growing processes must find those regions of neuropil where their targets reside. In this report we examine the growth of a late-developing neuron, the touch receptor AVM, in the nematode *Caenorhabditis elegans*. The AVM cell sends branches into the nerve ring, the major area of neuropil in the animal (*6*), where they synapse to interneurons needed for the touch reflex. Since the nerve ring is essentially complete at hatching, the late-arriving AVM branches must navigate through the mature neuropil of the nerve ring to contact appropriate targets.

Three touch receptors detect gentle touch to the head in *C. elegans* (*7*). Two of these cells arise embryonically, ALMR and ALML (the right and left ALM cells; Fig. 1) (*7*). The third cell, AVM, arises 10 hours after hatching and forms functional connections (via its branch) about 20 hours later (*7, 8*) (complete larval development takes 45 hours at 20°C). All three cells send branches into the nerve ring.

Since the reflex circuit requires the branches of the touch cells, the presence of the cells or their branches can be detected by testing for sensitivity to anterior touch [Table 1 (*7*)]. The bulk of anterior touch sensitivity derives from the ALM cells, since killing AVM with a laser microbeam does not significantly alter sensitivity to anterior touch ($n = 4$) (*7, 9*). When the ALM cells are killed at hatching, animals become only partially touch-sensitive as they mature as a result of the addition of AVM [Table 1 (*7*)].

A second postembryonic cell, PVM, is produced by a lineage homologous to that of AVM (*7, 8*). In adults the PVM cell body is located more posteriorly than AVM, and its process, which terminates before reaching the nerve ring, does not branch. PVM does not normally mediate a touch response. However, if PVM is positioned more anteriorly, as often happens in *mab-5* mutants, then the PVM process reaches the nerve ring, branches, and mediates a partial anterior touch response like AVM (*10*), even when AVM, ALMR, and ALML are absent. Thus, the presence of the branch appears to be position-dependent.

We assessed the effect of a pair of interneurons (the right and left BDU cells), which receive synapses from AVM (*6*), on touch cell development by killing the cells at various times with a laser microbeam and testing the resulting adults for touch sensitivity. Killing the BDU cells in the embryo (*11*) ($n = 3$) or at hatching ($n = 8$) had no effect on touch sensitivity, indicating that the BDU cells are not needed for the connections made by the embryonic ALM cells. Since the presence of the ALM cells would obscure any effect of BDU ablation on AVM development, we also examined animals in which the BDU and ALM cells were killed at hatching. These animals were completely insensitive to touch, despite the presence of AVM (Table 1), indicating that the BDU cells are needed for the AVM-mediated touch response.

The BDU neurons are required only during the early stages of AVM development. Killing the BDU cells 24 hours after hatching did not alter the AVM-mediated response; the resulting adults were partially touch-sensitive (Table 1 and legend to Fig. 2). These results and the fact that the BDU cells do not synapse directly onto any of the interneurons needed for backward movement (*6, 12*) make it unlikely that the BDU cells are a functional part of the AVM-mediated touch-reflex circuit. It seems more likely that the cells act to establish the circuit.

We achieved a genetic equivalent of the above laser ablations by using a temperature-sensitive mutation (*n848*) of the gene *unc-86*. Mutations in *unc-86* affect the lineages of the touch cells and the BDU cells and result in touch-insensitive animals (*13*). Shifting *unc-86(n848)* animals at hatching from the restrictive (25°C) to the permissive temperature (15°C) blocked the embryonic lineage that gives rise to ALM and BDU cells (the cells are sisters) but not the postembryonic lineage producing AVM. The resulting AVM cells, which differentiated in the presence of the undivided ALM-BDU precursor, did not mediate a touch response ($n = 12$).

Similar results were found in experiments with *mab-5* animals. In *mab-5* mutants the PVM cell is variably positioned anteriorly, and its process may or may not reach the nerve ring (*10*). Mutant animals in which the ALM cells were killed at hatching exhib-

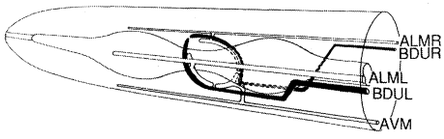

Fig. 1. Schematic diagram of the structural relation between the anterior touch cells and the BDU neurons in the nerve ring derived from a number of serial reconstructions (*6*). The processes of the three touch cells run in fixed positions near the surface of the animal. Each process branches and enters the nerve ring, where synapses are made with interneurons involved in touch-mediated movement as well as with the BDU cells. The anterior processes of the BDU cells are also shown, illustrating their close association with the touch cells in the nerve ring. AVM normally branches between 1 and 2 μm anterior to the excretory pore (not shown); the ALM cells branch at approximately 10 μm anterior to the pore (*6*).

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